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64 Process for encapsulation and encapsulated active material system(.

67 Capsules are formed having a liquid core while avoiding capsule core gelatin by adding drops of a solution of either an anionic polymer composition or a cationic polymer composition to a solution of an ionic polymer of opposite charge. The drops contain an active ingredient such as a cell or microorganism capable of producing a biologically active product or can contain a biological or chemical composition. The interface of the ionic polymers form a permeable membrane surrounding the liquid drops.

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6 Process for Encapsulation and Encapsulated Active  
Material System

This invention relates to a process for encapsulating  
biologically active materials such as cells or tissues  
10 or biochemically or chemically active compositions and  
to the encapsulated system including the active materials.

In biochemical production and biotechnological applicat-  
ions, health and viability of active materials such as  
15 cells, microorganisms and the like, is important since  
these active materials are capable of producing  
biologically or biochemically active components that find  
a wide variety of use. For example, cells are capable  
of producing antibodies, hormones, lymphokines, anti-  
20 biotics, interferons and other biochemicals or chemicals.  
Mammalian cell lines are grown by being surrounded by  
an aqueous medium containing a nutrient in order to  
promote the viability and growth of the cells and enables  
continued production of the desired microbiological or  
25 biological products. It has been proposed to utilize  
so-called microcarriers, which are beads having the  
appropriate charge and exchange capacity to promote  
the growth of the cells thereon in an efficient manner.  
The microcarriers themselves are maintained in an  
30 aqueous suspension containing the proper nutrient  
composition to promote cell growth and production of  
the desired microbiological product.

Biological products which are shed or excreted from the  
35 cells become admixed with the aqueous suspending

- 1 composition, which in many cases, is at very dilute concentrations. The subsequent recovery of the desired product is thereby rendered difficult and time consuming.
- 5 In order to overcome problems associated with microbiological product recovery, it has been proposed to encapsulate cells or microorganisms within a membrane which permits nutrients to be metabolized by the cell or microorganism while retaining the microbiological product produced by the cell or microorganism within the encapsulating membrane. Such processes are disclosed, for example, in U.S. Patents 4,409,331 and 4,352,883. The semipermeable membrane surrounding the biologically-active material has a selected permeability so that
- 15 substances having a certain molecular weight or below, are allowed to pass through the semi-permeable membrane. By controlling the permeability of the membrane, and by having a knowledge of the approximate molecular size of the desired product, one can confine the product,
- 20 within the space between the active material and the semi-permeable membrane.

Unfortunately, the process described in U.S. Patent 4,409,331 and 4,352,883 require that the membrane be

25 formed from the surface of an initially formed solid gel bead. This requires that the interior of the bead be subsequently liquefied so that the diffusion of nutrient which is required by the microorganism or cell, will not be hindered thereby to promote formation

30 of the desired microbiological product. Furthermore, liquefaction of the gel is highly desired so that the space between the semi-permeable membrane and the microorganism or cell is available for either cell production or products. Typically, these prior art

35 membranes are formed from a cell suspension in alginate

1 solution which is added dropwise to a calcium chloride  
aqueous solution, thereby to form solid gel beads. The  
beads then are washed with N-cyclohexylamine ethane  
sulfonic acid (CHES) and then washed subsequently with  
5 sodium chloride. Thereafter, a polylysine solution is  
added to form a polymer complex with the alginate  
surface. This surface then is washed with CHES/sodium  
chloride, subsequently with calcium chloride and then  
subsequently with sodium chloride. The membrane then  
10 is incubated and the gel within the membrane is  
subsequently liquefied by washing twice with sodium  
chloride, incubating, washing with sodium citrate and  
sodium chloride, washing with sodium chloride, and then  
a final wash. Obviously, such a process for forming  
15 encapsulated microbiologically active ingredients is  
time consuming and difficult and requires a high level  
of laboratory technique in order to successfully  
produce the encapsulated cell or microorganism suspended  
in a liquid medium. Furthermore, during these complicated,  
20 time-consuming steps, the viability, productivity or  
other characteristics of the cell may be altered.

It would be highly desirable to provide a means for  
encapsulating a microorganism or cell capable of  
25 producing a biologically active material which eliminates  
the necessity of liquefying a solid carrier in order  
to promote mass transfer into and out of the cell  
or microorganism. Furthermore, it would be desirable  
to provide such an encapsulating means which is capable  
30 of drastically reducing the number of steps needed to  
form the encapsulated cell or microorganism. In  
addition, it would be desirable to provide such an  
encapsulating means which permits the formation of  
a membrane capable of having a permeability over  
35 a wide range, which permits the isolation or selective

1 separation of a wide variety of biologically or chemically  
active molecules.

5 In accordance with this invention, cells, microorganisms,  
or the like, capable of producing a biologically active  
composition or biochemicals such as enzymes or hormones  
or the like or nonbiochemical compositions such as  
10 substrates, reactants, or catalysts are encapsulated by  
a polymer complex comprising the combination of an anionic  
polymer and a cationic polymer. The term "active mater-  
ial" is used herein to include cells, microorganisms or  
the like which produce a biologically active composition  
or a composition such as an enzyme, hormone, antibody,  
15 antibiotic insecticide, catalyst, substrate or reactant  
or the like which active material is to be encapsulated  
in accordance with this invention. The active material  
is suspended in an aqueous solution of either one of the  
cationic polymer or the anionic polymer composition.  
20 The polymer composition containing the active material  
then is formed into non gel liquid particles and is  
added to the other polymer such as in the form of drops  
from a capillary tube or a spray or the like to form  
capsules comprising a membrane surrounding a liquid core.  
25 The active material is housed within the interior of the  
membrane suspended in the liquid core. The capsules then  
are washed and ready to use or then can be stored in an  
appropriate medium until use. The permeability of the  
membrane is controlled by controlling concentration of  
30 the cationic and anionic polymers in the solution used  
in the preparation of the capsule, the pH of the aqueous  
solutions in which the cationic polymer or anionic  
polymer are prepared, the presence or absence of counter-  
ions in each solution, and the molecular weight of the  
35 anionic polymer and the cationic polymer as well as  
the selection of specific polymers.

1 The process of this invention eliminates the need for  
liquefying the core of the capsule containing the active  
ingredient and also eliminates the need for multiple  
washing steps with a variety of reagents which may  
5 adversely affect the biological, biochemical or chemical  
activity of the active ingredient to be encapsulated.  
In addition, the process of this invention is useful  
with a wide variety of biologically active molecules  
over a wide molecular type and weight range, since the  
10 permeability of the membrane formed around the capsule  
can be varied widely. Thus, it is possible to separate,  
isolate or selectively segregate biologically active  
compounds of varying nature by controlling the perme-  
ability of the membrane.

15 In accordance with this invention, an active material  
comprising or being capable of producing biologically  
active compositions is encapsulated within a membrane  
capable of permitting transport of a variety of compounds  
20 such as a nutrient for a cell to the active material  
and capable of selectively containing, within the mem-  
brane, the compound produced. The active ingredient can  
be a cell, microorganism, tissues or chemical or bio-  
chemical reactants. Representative suitable cells  
25 include fused cells, eg. hybridoma cells, or genetically  
modified cells produced by recombinant DNA technology  
and lymphocyte cells capable of producing antibodies  
or microorganisms for fermentation.

30 In addition, microorganisms such as bacteria, can be  
encapsulated in accordance with this invention.  
Furthermore, biologically active compositions such as  
enzymes, hormones, <sup>antibiotics,</sup> antibodies or the like can be  
encapsulated so that they can be controllably released  
35 through the membrane or retained therein if desired.

1 The encapsulated active ingredient is enclosed by the  
membrane, which also can contain an aqueous medium which  
includes nutrients for the active ingredient. The  
aqueous medium also is capable of dissolving or sus-  
5 pending the microbiologically active material produced by  
the active ingredient without degrading it. The perme-  
ability of the membrane is such as to permit passage  
of nutrients from a medium surrounding the membrane into  
the aqueous medium enclosed by the membrane, and so that  
10 the microbiologically active composition can be produced  
by the active ingredient.

The active ingredient first is suspended in an aqueous  
solution of either (a) one or more anionic polymers or  
15 (b) one or more cationic polymers. The anionic polymers  
or cationic polymers chosen are formed of molecularly  
repedative segments linked together which here are  
either positively charged or negatively charged segments  
distributed along the chain or on substitutions distribut-  
20 ed along the chain. The concentration of charged segments  
is such as to permit electrostatic interaction and en-  
tanglement of the polymers when they are contacted  
together thereby to form the membrane. The resultant  
suspension then is sprayed into or added dropwise or the  
25 like as liquid particles to the other polymer so that  
a membrane is formed at the interface between the  
anionic polymer and the cationic polymer. When the  
interface between the two oppositely charged polymers  
encloses the active ingredient, the active ingredient  
30 thereby becomes encapsulated. Representative suitable  
anionic polymer include alginate, carragenen,  
hyaluronic acids, carboxymethylcellulose, xanthan,  
furcellaran and, sulfonated organic polymers, usually  
in salt form, eg, sodium salt. Representative suitable  
35 cationic polymers include chitosan, polylysine,

1 polyethylamine and polyvinylamines as well as other  
amine or imine containing polymer which is capable of  
coacting with an anionic polymer to form a membrane. The  
preferred anionic polymers are alginate, or carragenan.  
5 The preferred cationic polymers are chitosan, or poly-  
lysine. The droplets of the charged polymer containing  
the active ingredient can be regulated in order to  
regulate the size of the final encapsulated product.  
Typical encapsulated products have a size within the  
10 range of about 50 microns and 5000 microns. When cells  
are to be encapsulated, the capsule has a size which  
permits oxygen transfer to those cells that require  
oxygen for vitality and has a size sufficiently small  
to afford efficient isolation of the desired cell product,  
15 typically between about 400 and 800 microns.

The permeability of the membrane formed by the inter-  
action of the anionic polymer and the cationic polymer  
is controlled by controlling the relative concentration  
20 of the two oppositely charged polymers, their concentrat-  
ion in the individual aqueous media, the pH of each of  
the polymer solutions, the molecular weights of the  
polymers and presence or absence of counter-ions in  
either of the solutions. By the term "counter-ions" is  
25 meant ions which interact with the charged portion of  
the polymer in order to reduce interaction of that  
polymer with the oppositely charged polymer. For  
example, calcium ion interacts with carboxyl ion on  
the anionic polymer. The calcium ion can be removed  
30 with phosphate ion. Increased polymer concentration  
usually results in decreased permeability. A decrease  
in the pH of the anionic polymer composition results  
in increased concentration of hydrogen ion thereby to  
form reactive cations on the cationic polymers having  
35 amine or imine groups. The achievement of a membrane

1    having a desired permeability can be determined by  
varying the process parameters and incorporating a  
mixture of compounds of anions molecular weight and size  
in the droplets or spray. The aqueous medium outside the  
5    capsules thus produced can be assigned for the presence  
of these compounds so that the molecular weight/molecular  
size cut-off level of the membrane is thus determined.

This invention also provide capsules having a normal  
10    membrane structure having improved mechanical properties  
as compared to the capsules of the prior art. Membranes  
produced from a gel composition and which are subsequent-  
ly liquified have reduced strength. This is due primarily  
to the fact that a large proportion of the polymer chains  
15    becomes oriented towards the interior of the capsule  
during gelation rather than in the plane of the membrane.  
During liquification of the gel, these portions of the  
polymer chain do not become reoriented into the plane  
of the membrane and therefore do not contribute to  
20    membrane strength. In contrast, in this invention, the  
ionic portions of the anionic and cationic polymers need  
not be encumbered with counter ions so that they are  
free to react with each other along the entire chain  
length where the different polymers come into reactive  
25    contact. By operating in this manner, larger chain  
lengths of the polymers are oriented in the plane of  
the membrane. In one particular aspect of this invention,  
it is possible to have the anionic polymer oriented  
on the outside of the membrane rather than on the inside  
30    of the membrane. Thus, for example, alginate can  
comprise the outer membrane surface. The result is not  
possible with prior art processes since the alginate  
is required to form the initial gel bead. Thus, this  
invention provides the user with much greater flexi-  
35    bility in forming the capsule. In another particular

- 1 aspect of this invention, multi-membrane walls can be  
formed thereby providing membranes with greater strength  
as compared to capsule of the prior art. This is  
accomplished by forming the capsule with the anionic  
5 polymer chain on the outside of the membrane by the  
process set forth above. The capsules then are separated  
from the surrounding aqueous medium by any convenient  
method such as filtration or centrifugation. The capsules  
then are mixed with a solution of anionic polymer and  
10 a crosslinking divalent metal ion. For example, in the  
case of alginate as the anionic polymer, calcium ion or  
barium ion can be used as the crosslinking divalent ion  
to form an outer membrane of alginate polymer.
- 15 After the encapsulated active ingredients are produced  
in accordance with the above-described process, then  
they can be separated from the aqueous medium where  
they are suspended, and then reintroduced into an aqueous  
medium which contains the nutrients for the active  
20 ingredient, so that the microbiologically active  
compound can be produced. On the other hand, the  
nutrients can be added to the suspension of encapsulated  
active ingredients without prior separation thereof.
- 25 The following examples illustrate the present invention  
and are not intended to limit the same.

Example I

- 30 An alginate solution comprises 0.75 percent--1 percent  
w/v sodium alginate and 150 mM NaCl was added dropwise  
to a chitosan solution. The chitosan solution comprised  
0.05--0.10 gr/dl chitosan, 117 mM NaCl, 0.01 M  $\text{CaCl}_2$   
and 0.01 M HCl. The chitosan solution had a pH of  
6.5. The alginate solution was added dropwise to the  
35 chitosan solution to form capsules which were incubated

1 in the chitosan solution for about one minute. Samples  
of the chitosan solution containing the capsules were  
separated by centrifugation or by filtration on a  
centered glass filter, washed with water and transferred  
5 separately to a phosphate buffer solution, a saline  
solution, distilled water or a cell culture medium  
comprising Dulbecco's Modified Minimum Essential  
Medium, 5 % Fetal Calf Serum and 5 % Calf Serum and were  
found to be surprisingly stable. In addition, the cap-  
10 sules were found to be able to sustain centrifugation  
at a level at least as high as about 2000 RPM for 10  
minutes. In this example, it is preferred that the  
alginate solution have a viscosity higher than about  
3.0 centistokes while a chitosan solution preferable  
15 has a viscosity of at least about 1.5 centistokes.

#### Example II

Following the procedure of Example I, capsules were  
formed by adding a chitosan solution dropwise to an  
20 alginate solution. The chitosan solution comprised  
1.5 percent w/v chitosan, 2.5 percent citric acid and  
0.07 M  $\text{CaCl}_2$ . The alginate solution comprised either  
1.1 percent w/v sodium alginate and 0.5 percent sodium  
sulfat, or a solution comprising 1 percent w/v sodium  
25 alginate. As in Example I, the capsules were found to  
be stable in phosphate buffer, saline, water and cell  
culture medium, and were able to sustain centrifugation  
at a level of about 2000 RPM for at least 10 minutes.  
The core of the capsules is rendered more fluid-like  
30 and less solid-like by lowering the concentration of  
calcium chloride in the chitosan solution.

#### Example III

35 Following the procedure of Example I, capsules were  
formed by adding chitosan solution dropwise in an

1 alginate solution to obtain capsules with a liquid core.  
The chitosan solution utilized contained between 0.1  
percent and 1.5 percent w/v chitosan, 0.05 M NaCl and  
between 0.006 M and 0.2 M  $\text{CaCl}_2$  and a pH rate ranging  
5 between 5.5 and 6.6. The alginate solution ranged between 0.1 percent and 1.0 percent sodium alginate.

As in Examples I and II, the capsules produced were found  
to be stable in phosphate buffer, saline, water and in  
10 the cell culture medium. As shown in Table I, the  
rupture strength of the capsules produced by this  
invention can be increased by treating them with a  
divalent ion after they are formed. Alternatively, the  
divalent ion can be added with an ionic polymer with  
15 which it does not interact to form a gel. The diffusion  
properties can also be controlled by solution conditions  
which influence the molecular configuration or the  
charge density of the polymers. The rupture strength  
of various capsules made in accordance with the  
20 procedures set forth in Example III is shown in Table I.

Table I Effect of divalent cations on the rupture strength of the capsules

25	Cation	Capsule Preparation	Rupture Strength g/cm <sup>2</sup>
	$\text{Ca}^{+2}$	1.35 % chitosan, 0.05 N in 0.5 % alginate; no treatment of capsules	8
	$\text{Ca}^{+2}$	Prepared as above + treatment of capsules in 0.1 M $\text{CaCl}_2$ for 5 minutes	743
30	$\text{Ba}^{+2}$	1.35 % chitosan, 0.05 M $\text{BaCl}_2$ dropped in 0.5 % alginate; no further treatment	696
	$\text{Ba}^{+2}$	Prepared as above + treatment of capsules in 0.1 M $\text{BaCl}_2$ for 5 minutes	1609
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15   C l a i m s

1. A process for producing a capsule having a liquid  
core from ionic polymers while avoiding gelation  
of said core which comprises:  
20   Forming a liquid droplet from an aqueous solution  
of a first ionic polymer selected from the group  
consisting of at least one anionic polymer and at  
least one cationic  
polymer and by contacting said droplet with a  
25   solution of at least one second ionic polymer, said  
second ionic polymer having an ionic charge opposite  
from said first ionic polymer and reacting said  
first and second polymer in contact with each other  
thereby to form a membrane encapsulating said  
30   droplet.
2. A process of claim 1 wherein said droplet contains  
an active material wherein said active material  
may be one of the group consisting of living cells  
35   or microorganisms or hybridoma cells or lymphocytes

- 1 or bacteria or a biologically active compound or  
an enzyme or a hormone.
- 5 3. A process for producing a product from a living cell  
which comprises encapsulating said cell by the  
process of claim 1 and controlling the permeability  
of said membrane to prevent said product from  
10 permeating said membrane, collecting said encapsulated  
cell, rupturing said membrane and recovering said  
product, wherein said cell may be one of the group  
consisting of a hybridoma or a lymphocyte cell.
- 15 4. A process of claim 1, 2 or 3, wherein said anionic  
polymer is selected from the group consisting of  
alginate and carragenan and said cationic polymer  
is selected from the group consisting of chitosan  
and polylysine.
- 20 5. A process of anyone of claims 1 to 4, wherein a  
second membrane is formed by cross-linking a second  
anionic polymer to the anionic polymer portion of  
said first polymer by adding a divalent metal cation  
and said second anionic polymer after said first  
25 membrane is formed, said metal cation may be  
selected from the group consisting of calcium,  
barium and mixtures thereof.
- 30 6. A capsule comprising a polymeric membrane surrounding  
a liquid core wherein said membrane is formed by the  
interaction of at least one anionic polymer with at  
least one cationic polymer and wherein molecular  
chains comprising said polymer are oriented  
35 substantially within said membrane.

- 1 7. The capsule of claim 6, wherein said cationic polymer forms the inner surface of said membrane adjacent liquid core.
- 5 8. The capsule of claim 6, wherein said anionic polymer forms the inner surface of said membrane adjacent said liquid core.
- 10 9. The capsule of claim 6, wherein said liquid core contains an active material, wherein said active material may be one of the group consisting of living cells, hybridoma cells and lymphocyte cells.
- 15 10. The capsule of anyone of claims 6 to 9, wherein said anionic polymer is selceted from the group consisting of alginate and carragenan or wherein said cationic polymer is selected from the group consisting of chitosan and polylysine.
- 20 11. The capsule of anyone of claims 6 to 10, which includes a second membrane formed by cross-linking the anionic polymer to the anionic polymer portion of said polymeric membrane with a divalent metal ion, wherein said divalent metal ion may be selected from the group consisting of calcium, barium and mixtures thereof.
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